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„A comparative study of the adhesive in different species
of Cirripedia Thoracica (Crustacea)“

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1. Abstract

The proteinaceous barnacle cement consists of a fibrous component embedded in a matrix. This led to the suggestion of the cement as a fibre-reinforced composite material. Previous studies measured the diameter of fibrillar and globular structures of the adhesives between 11 nm and 50 μm . Further it was reported that these fibrillar and globular structures can merge and form e.g. “net-like” and “sponge-like” structures. Also differences in the structure of the adhesive on different substrata were found. These investigations on the structure of the barnacle adhesive mainly focused on the group of Sessilia using atomic force microscopy (AFM) and scanning electron microscopy (SEM). Only few investigations were made on the adhesive of Pedunculata.

The aim of this study is to describe the structure of the cement of three pedunculate Cirripedia (the cyprid and dwarf male of *Scalpellum scalpellum*, the dwarf male of *Ibla cumingi* and the hermaphrodite of *Pollicipes pollicipes*) by light- and transmission electron microscopy. Different fibre-thickness of the species analysed were measured. The orientation of the fibres within the cement of the species analysed differed from each other. This implies different physical properties of the adhesives according to the different environmental conditions the animals are exposed to such as e.g. substratum and water movement. It is obvious, that only the cement of *Pollicipes pollicipes*, living in the surf, shows clear zonation, compared to the cement of the other species analysed that live protected.

2. Introduction

The order of Thoracica within the Cirripedia (a subclass of the Crustacea), is separated into the suborders of the Sessilia (acorn barnacles) and the Pedunculata (stalked barnacles). The body of the Pedunculata is divided into a capitulum, covered by calcareous plates, and a peduncle or stalk. The body of the Sessilia has a broad basis which is cemented to the substratum and no peduncle (Newman & Abbott, 1980).

Most studies of the cement apparatus (morphological and histochemical) were accomplished on balanoid Sessilia in the late 1960s and 70s (Karande & Gaonkar, 1977; Lacombe, 1970; Lacombe & Liguori, 1969; Walker, 1970; Walker, 1978) and only little work has been done on Pedunculata (Jonker et al., 2012; Klepal, 1985; Lacombe & Liguori, 1969; Zheden et al., 2012).

The cement apparatus consists of the cement producing glands that are unicellular in adult barnacles (Lacombe & Liguori, 1969) and multicellular in cyprids (Walker, 1971). The mature glands are ovoid in shape and in their nuclei they contain a lot of nucleoli revealing them as highly active cells (Zheden et al., 2012) that secrete the proteinaceous adhesive (Lacombe & Liguori, 1969; Saroyan et al., 1970). The cement glands are localized close to the ovaries in the stalk of Pedunculata. In the cyprid of the acorn barnacle *Balanus balanoides* the cement glands are kidney-shaped and lie posterior to the compound eyes. They contain two cell-types (α - and β - cells) (Walker, 1971).

It was reported from the adult acorn barnacle *Balanus tintinnabulum* that cement glands can appear singly or in groups of up to 20 (Lacombe & Liguori, 1969). Similar results were shown by Karande & Goankar (1977) in *Balanus kondakovi* where the gland cells appear mostly singly or in pairs but also in groups of up to six cells. In the pedunculate *Pollicipes pollicipes* for instance the cement glands are arranged in rosettes of five to ten cells (Mc Evilly, 2011). An efferent duct system leads from the gland cells to the base plate of acorn

barnacles, respectively to the attachment disc of the peduncle in stalked barnacles where the cement is extruded (Lacombe & Liguori, 1969). It is suggested that “the degree of development and differentiation in the cement gland system may be related to the phylogenetic position of the species” (Lacombe, 1970; p.177).

Three types of cement can be distinguished depending on the time when the adhesive is secreted - the larval cement, the primary and secondary cement. Primary cement is the adhesive secreted by mature barnacles for attachment whereas secondary cement is only extruded when the barnacle is injured or detached (Saroyan et al., 1970).

The main focus of attention in this work is to describe the cement of three Pedunculata, the cyprid of *Scalpellum scalpellum* (LINNAEUS, 1767), the dwarf males of *Scalpellum scalpellum* and *Ibla cumingi* (DARWIN, 1851) as well as the hermaphrodite of *Pollicipes pollicipes* (GMELIN, 1790), by light microscopy (LM) and transmission electron microscopy (TEM) since little research has been done on that topic. In the past structural analysis of the cement was made using mainly atomic force microscopy (AFM) and scanning electron microscopy (SEM) (Berglin & Gatenholm, 2003; Sullan et al., 2009; Wiegemann & Watermann, 2003).

On the one hand it will be discussed if the systematic positions of the species analysed influences the structure of the cement. On the other hand the different ways of life and living conditions of the animals are considered in the analysis.

3. Material and methods

3.1. Habitat and occurrence of the animals investigated

Scalpellum scalpellum inhabits sublittoral zones in the eastern north Atlantic. It occurs in depths between 30 m – 200 m. The hermaphrodites are mainly found growing on hydroids (Buhl-Mortensen & Hoeg, 2006), the cyprids (Fig. 1a) can also grow on hydroids, further in the receptacles, at the rim of the mantle aperture or on the external surface of the hermaphrodite. The dwarf males of *Scalpellum scalpellum* (Fig. 1b) only occur in the receptacles of the hermaphrodite (Spremborg et al., 2012). The hermaphrodite of *Pollicipes pollicipes* (Fig. 1c) is an intertidal cirripede. It is found between the west coast of Africa (15°N) and the Atlantic coast of France and “favours exposed habitats where there is a backwash from surging waves” (Barnes, 1996; p.303). *Ibla cumingi* is also native to intertidal zones in the Gulf of Elat amongst other regions. The dwarf male of *Ibla cumingi* (Fig. 1d) adheres to the female tissue in the mantle cavity where it lives well protected (Klepal, 1985).

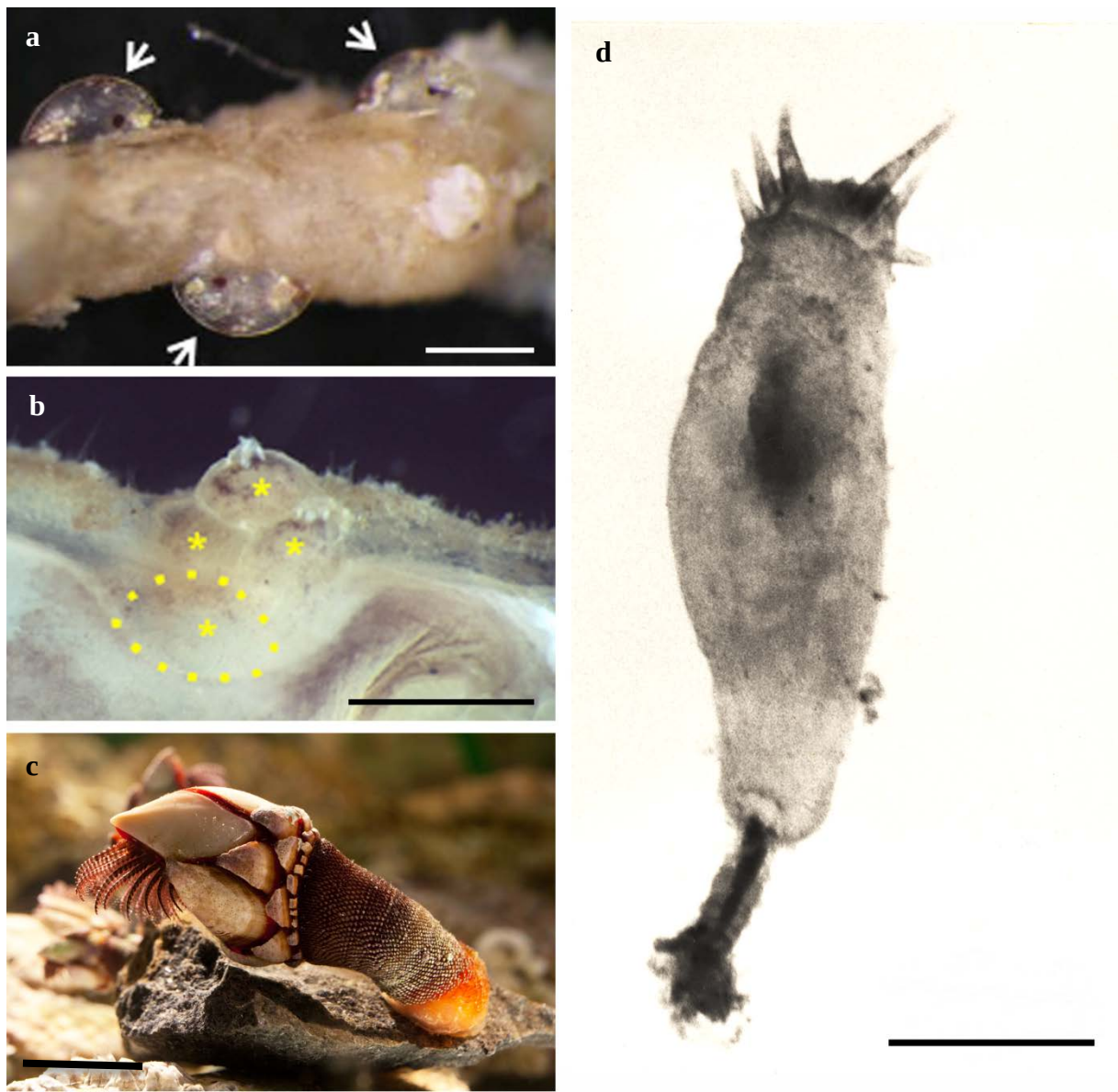


Fig. 1: Pictures of the species analysed. *Scalpellum scalpellum*: **a)** arrows show cyprids (Spremberg et al., 2012; supplementary Fig.3d; <http://dx.doi.org/10.1016/j.jembe.2012.04.004>), **b)** dwarf male (Spremberg et al., 2012; page 43; Fig.4e), asterisks indicate males. **c)** *Pollicipes pollicipes* (picture by Gregor Eder). **d)** *Ibla cumingi* dwarf male (Klepal, 1985; page 53, Fig.3a). Scale bars a, b: 500 μ m; c: 1 cm; d: 0.5 mm.

3.2 Systematic position of the animals investigated

Ibla cumingi belonging to the monophyletic suborder of the Iblomorpha has a basal position within the Thoracica and represents the sister-group to all the other Thoracica. *Pollicipes pollicipes* and *Scalpellum scalpellum* belong to the polyphyletic suborder of the Scalpellomorpha and *Pollicipes* seems to be higher evolved than *Scalpellum* (Pérez-Losada et al., 2008).

3.3. Cement

It can be assumed that the cement of the adult *Pollicipes* and the dwarf males of *Scalpellum* and *Ibla* is primary cement and that of the *Scalpellum* cyprid must be larval cement. (Saroyan et al., 1970).

3.4 Fixation and analysis

The material used for the present study was prepared by various students (Alexandra Kerbl, Nikolaus Leisch, Johannes Suppan and Vanessa Zheden) of the *Core Facility of Cell Imaging and Ultrastructure Research* during the period of three years (2010 –2013). All the LM and TEM investigations in this study were carried out on these pre-prepared sections.

Countries of origin of the species and dates of embedding are listed in Tab. 1.

Tab. 1: Origin and preparation of the specimen.

Species	Location	Preparation
<i>Ibla cumingi</i>	Gulf of Elat, Israel	2012
<i>Pollicipes pollicipes</i>	Cabo de Sines, Portugal	2010
<i>Scalpellum scalpellum</i>	Denmark (preserved in formalin since 1930)	2013

The samples of *Ibla cumingi* were fixed with glutaraldehyde (GA) + Sorensen`s Phosphate (PBS buffer), those of *Scalpellum scalpellum* with GA + Na-Cacodylate buffer and those of *Pollicipes pollicipes* were fixed in Karnovsky-fixative + Na-Cacodylate buffer. The samples of *Scalpellum scalpellum* were previously kept in formalin since 1930. After fixation with GA and decalcification of *Scalpellum scalpellum* with EDTA (10%) for 45 minutes, all specimen were rinsed three times á 10 minutes with the respective buffer. The post-fixation was made with osmium tetroxide (OsO₄) for 2 hours followed by an upgrading ethanol series for dehydration. Infiltration was carried out with acetonitril and ethanol (100%) 1:1 for 10 minutes. After rinsing twice for 10 minutes with acetonitril infiltration with a mixture of acetonitril and Agar low viscosity resin (LVR) respectively with Epon for *Pollicipes* began in proportion 1:3 and 1:1 for 3 hours each. After that step the acetonitril evaporated overnight.

Then the samples of *Ibla* and *Scalpellum* were transferred into pure LVR, and those of *Pollicipes* into Epon. Samples of *Scalpellum* were put into a desiccator for 15 minutes to avoid air bubbles in the resin. Then the specimen were embedded and pre-polymerized at 40°C for 3 hours in *Scalpellum* and *Ibla*, and overnight in *Pollicipes*. Finally the polymerization at 60°C was finished after 20 hours in *Scalpellum*, 3 days in *Pollicipes* and 2 days in *Ibla*.

Ultrathin sections (60 nm) were made with the microtomes Reichert Ultracut E and S and the Leica EM UC7. Pictures of the unstained sections were taken on the transmission electron microscope Phillips EM 208. Semithin sections (1 µm) cut with the same microtomes were stained with toluidine blue capped with Agar LVR and analysed with an Olympus BX 41 light microscope equipped with a Olympus Coler View III camera. The edition of the pictures (cut to size, scale bars) was performed with the software program Adobe Photoshop CS5. The measurements of the fibres were carried out with the software program iTEM, mean values and standard deviations were calculated.

4. Results

4.1 Structure of the cement

4.1.1 Light microscopy

The antennules of the cyprid and the dwarf male of *Scalpellum scalpellum* are embedded in cement and surrounded by the tissue and the cuticle of the hermaphrodite (Fig. 2a, 2c). At higher magnification differently stained regions of the adhesive are obvious (arrows in Fig. 2b, 2d). The antennules of the dwarf male of *Ibla cumingi* are surrounded by the female tissue and the cuticle of the mantel cavity (Fig. 2e) and are likewise embedded in cement. Three differently stained regions within the adhesive and inclusions of microorganisms within the cement are seen (Fig. 2f). In contrast the cement of *Pollicipes pollicipes* (Fig. 2g) appears clearly layered (Fig. 2h).

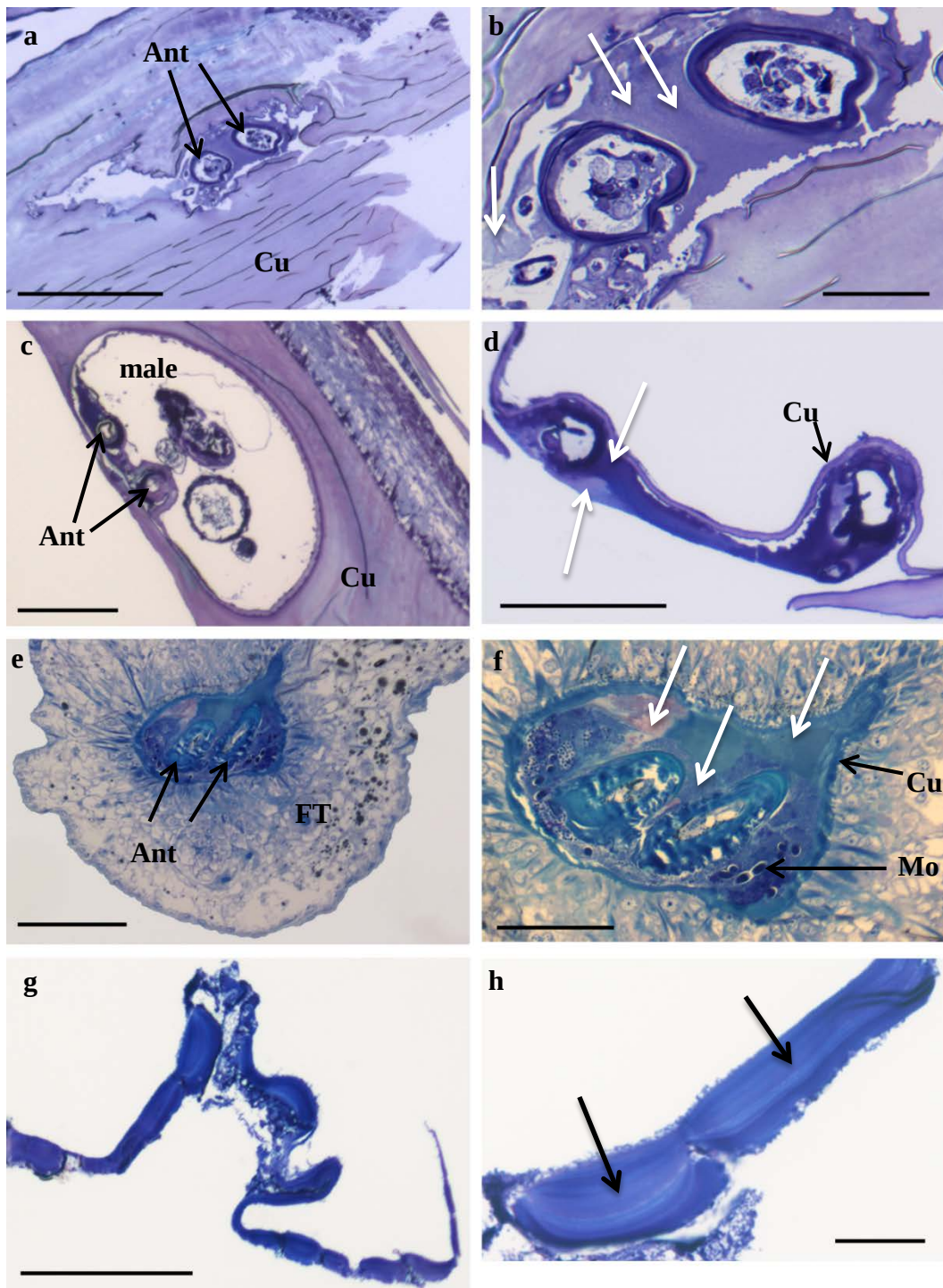


Fig. 2: Overviews (left column) and details (right column) of the cement of the species analysed. **a, b)** Cyprid of *Scalpellum scalpellum*. Antennules (a) are embedded in cement (white arrows in b), surrounded by cuticle of the hermaphrodite. **c, d)** Dwarf male of *Scalpellum scalpellum*. Section through the dwarf male (c) and detail of the antennules embedded in cement (white arrows in d). **e, f)** Dwarf male of *Ibla cumingi*. Antennules embedded in cement (white arrows in f), surrounded by female tissue and cuticle. **g, h)** Cement of the hermaphrodite of *Pollicipes pollicipes*. Layers of cement are perpendicular to the arrows (h). Ant...antennules, Cu...cuticle, FT...female tissue, Mo...microorganisms. Scale bars a, c, e, g: 100 μ m; b, h: 20 μ m; d, f: 50 μ m.

4.1.2 Transmission electron microscopy

In all species investigated so far the cement consists of a matrix in which fibres are included. In the following part the structure of the cement of the cyprid of *Scalpellum scalpellum*, the dwarf males of *Scalpellum scalpellum* and *Ibla cumingi* as well as from the hermaphrodite of *Pollicipes pollicipes* are described.

4.1.2.1 The cyprid of *Scalpellum scalpellum*

The structure of the fibres in the cement that is surrounding the antennule of the cyprid is akin a meshwork. Two areas can be distinguished that differ in the density of the fibres (Fig. 3). In the dense area of the cement the fibres are fused to a tight network so that the matrix can hardly be seen (Fig. 4a, b). In the less dense area more matrix is obvious and single fibres are enclosed (Fig. 4c). Both areas of dense and less dense cement are close to the cuticle of the antennules. Where the cuticle of the hermaphrodite forms “foot-like” elevations the detached cement of the cyprid forms an undulated edge (Fig. 5).

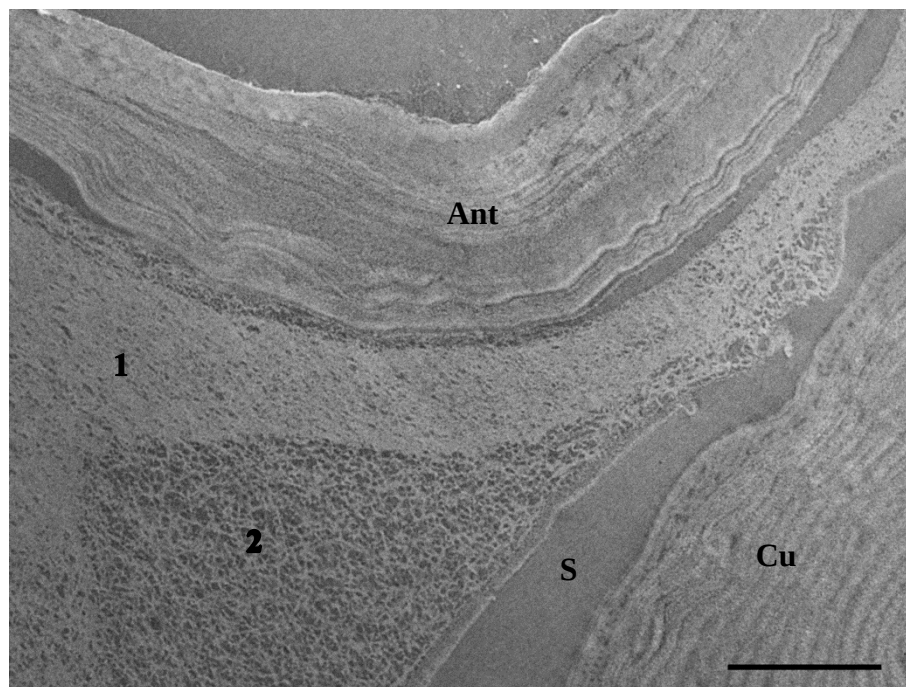


Fig. 3: Cement of the cyprid of *Scalpellum scalpellum*. Meshwork-like cement between the antennule (Ant) of the cyprid and the cuticle (Cu) of the hermaphrodite. Dense fibres (1) and loose fibres (2) of the cement. The adhesive is partly detached from the cuticle. S...space between cuticle and cement (due to detached cement). Scale bar: 2 μm .

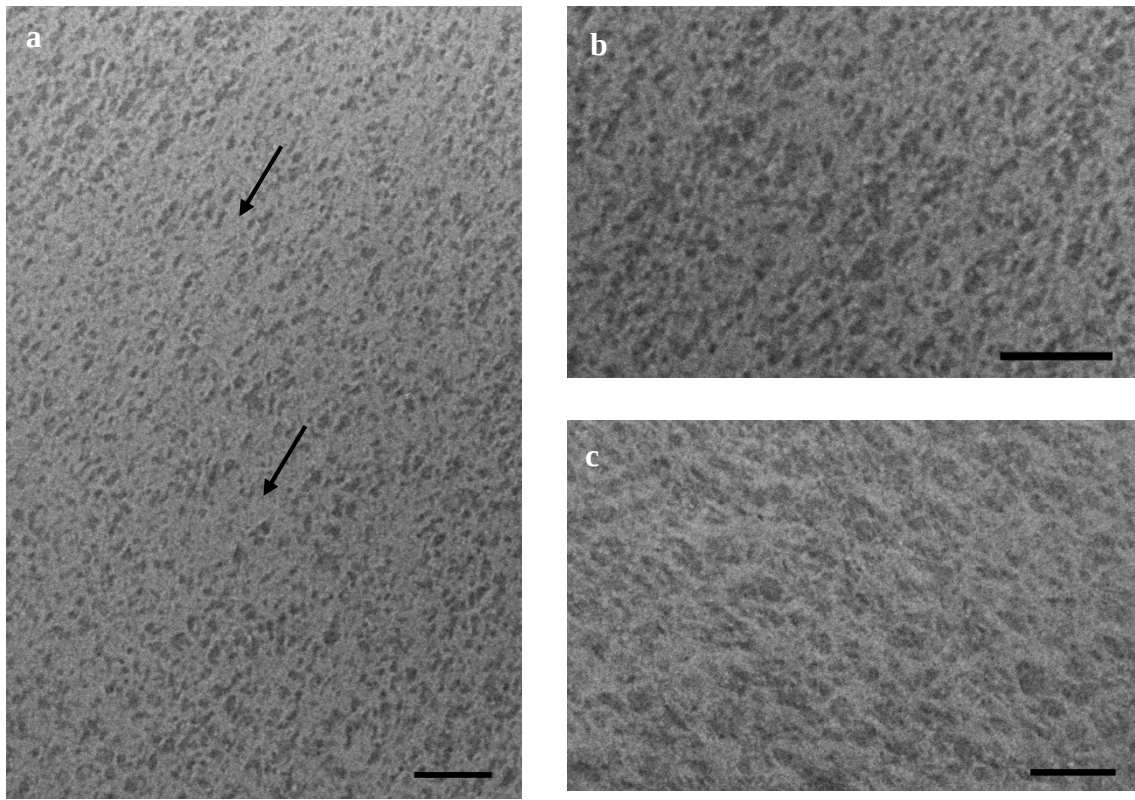


Fig. 4: Comparison of the two areas observed in the cyprid cement of *Scalpellum scalpellum*. **a)** Dense area with tightly packed fibres (Arrows). **b)** Dense area with looser fibres. **c)** Less dense area where fibres form a loose reticulum. Scale bars a, b, c: 0.5 μ m.

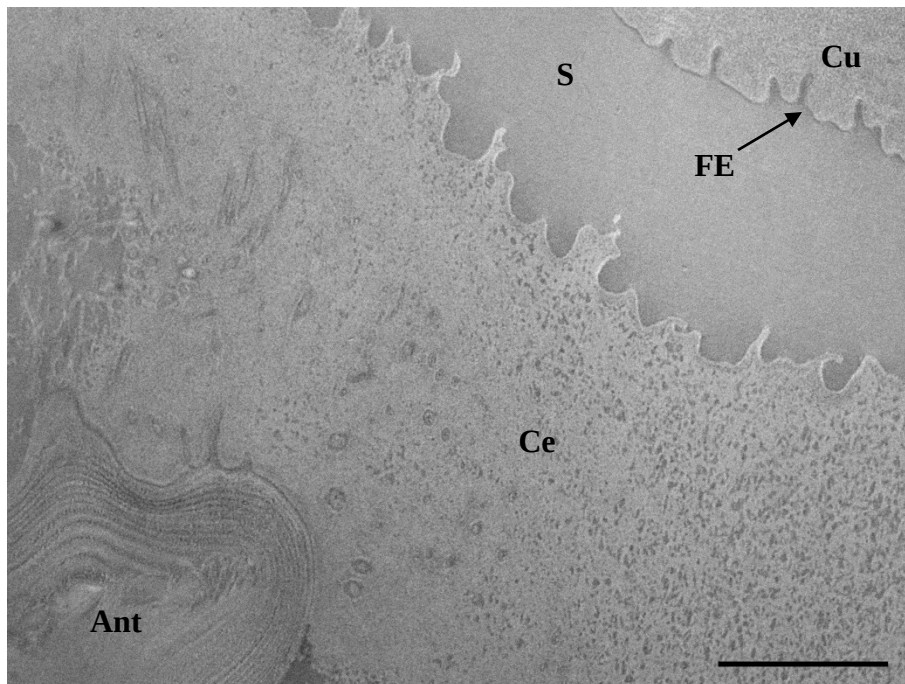


Fig. 5: Cement (Ce) of the cyprid of *Scalpellum scalpellum* surrounding the antennule (Ant) of the cyprid. In the upper right corner the cuticle (Cu) of the hermaphrodite with “foot-like” elevations (FE) is seen and the detached cement forming an undulated edge. S...space between cuticle and cement. Scale bar: 2 μ m.

4.1.2.2 The dwarf male of *Scalpellum scalpellum*

The structure of the cement in the dwarf male of *Scalpellum scalpellum* is similar to that in its cyprid (Fig. 6-8). As the cement is extruded at the base of the antennule, the latter is surrounded by the adhesive (Fig. 6). Concerning the denseness of the cement there does not seem to be a specific area surrounding the antennules of the male of *Scalpellum scalpellum*. Fibres form a meshwork of varying density (Fig. 7, 8a). In some particular places it looks like parallel layering as indicated in Fig. 7 but this is not in the entire object. Again the cement can be divided into two areas – a dense and a less dense area (Fig. 8b, c). Closer inspection shows in the dense area tight fibres with little interspace (Fig. 8b) and in the less dense area a loose reticulum of fibres (Fig. 8c). The cuticle of the male also shows “foot-like” elevations (Fig. 7) and the detached cement sometimes forms an undulated edge (Fig. 6).

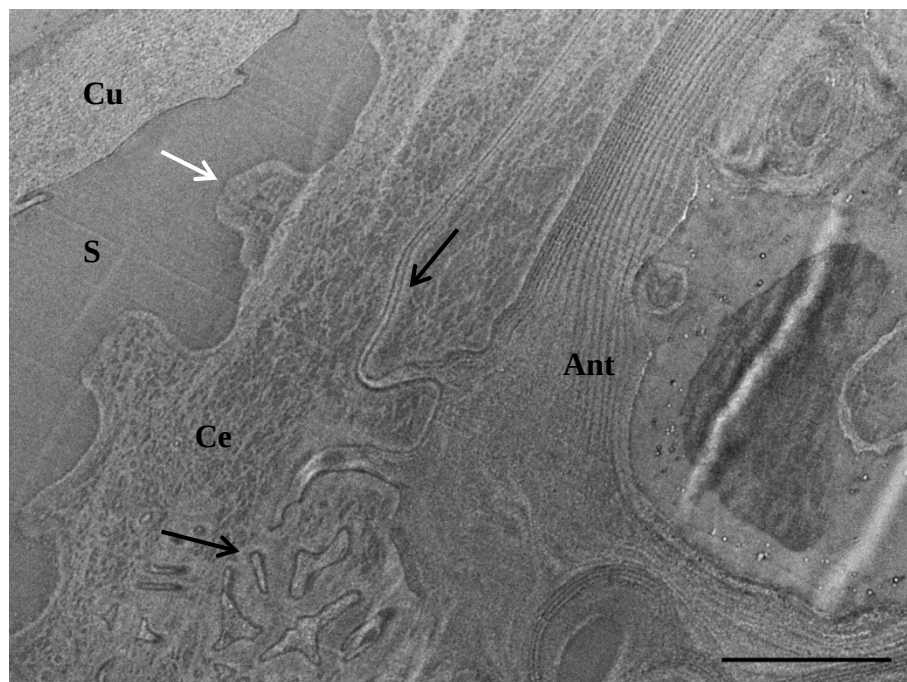


Fig. 6: The antennule (Ant) of the male of *Scalpellum scalpellum* is surrounded by cement (Ce). Cuticular extensions of the antennule (black arrows) reach into the adhesive. At the left side of the picture the cement is removed from the cuticle (Cu) of the male forming an undulated edge (white arrow). S...space between cuticle and cement (due to detached cement). Scale bar: 2 μ m.

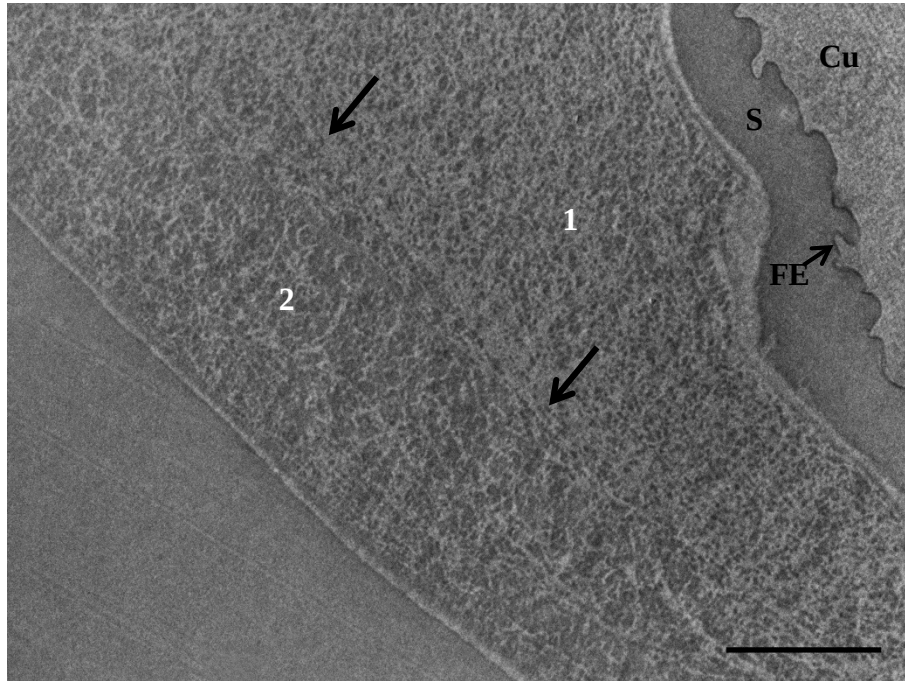


Fig. 7: *Scalpellum scalpellum* male. The cement is removed from the cuticle (Cu) that forms “foot-like” elevations (FE). Clear border between the densely arranged fibres (1) and less dense arranged fibres (2) of the cement (arrows). S...space between cuticle and cement (due to detached cement). Scale bar: 2 μ m.

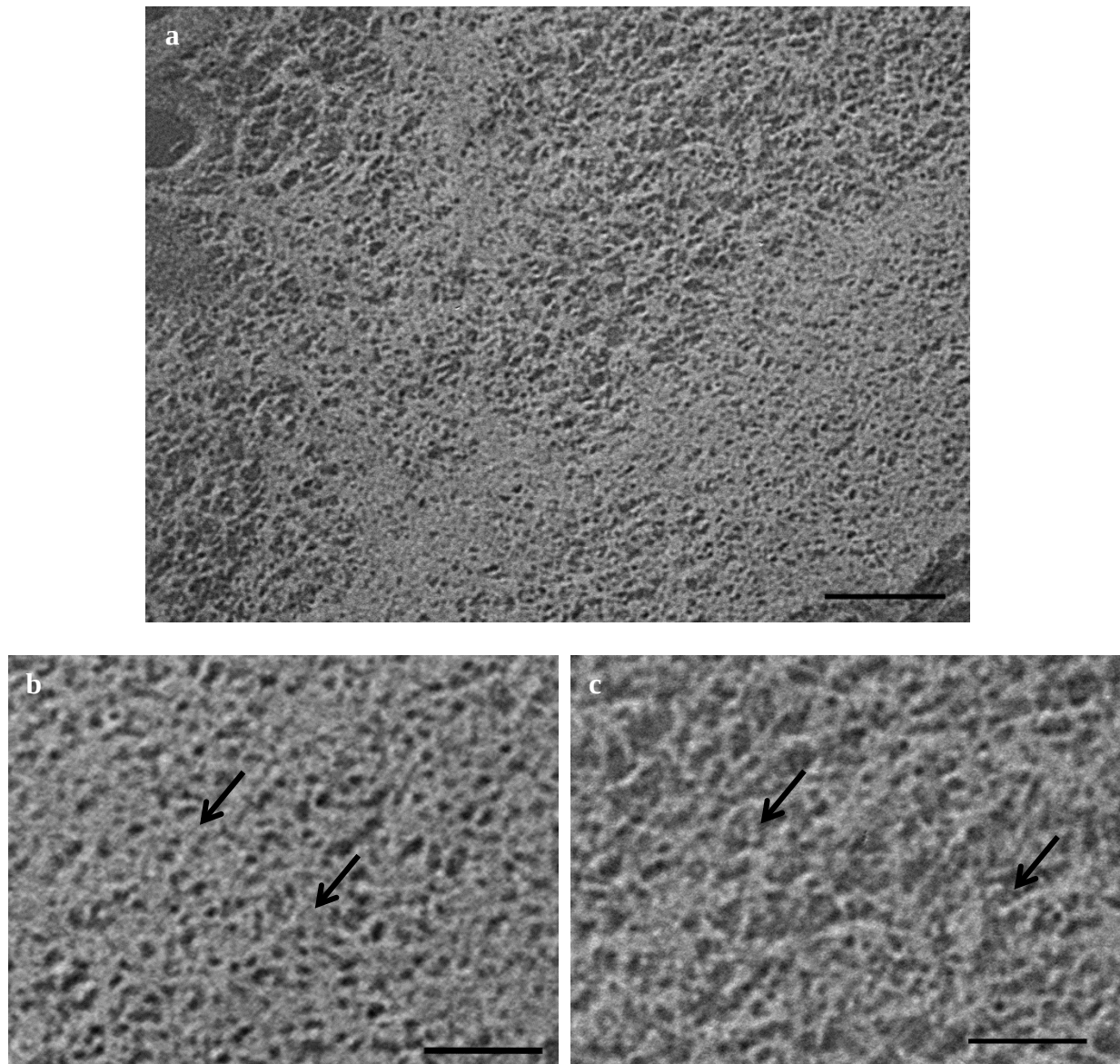


Fig. 8:. Comparison of the two areas observed in the cement of the male of *Scalpellum scalpellum*. **a)** Overview of the cement. **b)** Densely arranged fibres (arrows). **c)** Less dense arranged fibres (arrows). Scale bars a: 1 µm; b, c: 0.5 µm.

4.1.2.3 The dwarf male of *Ibla cumingi*

In some regions of the cement of the male of *Ibla cumingi* the fibres are more clearly seen than in others where they appear less electron-dense (asterisks Fig. 9). This is because of the different orientation of the fibres within the cement. Therefore three regions can be distinguished that are distributed in patches (Fig. 10). The 1st area is interspersed with inhomogenously arranged electron-dense fibres surrounded by less electron-dense fibres and a bright electron-lucent matrix (Fig. 11, 12, 14a, 14b). The fibres of the 2nd area appear more homogenously distributed and the majority is longitudinally sectioned (Fig. 11, 13, 14c, 14d).

In contrast, the 3rd area predominantly contains electron-dense granular dots that are cross sections of the fibres. They are homogenously distributed and intermingled with few fibres that are longitudinally sectioned (Fig. 11-13, 14e). Longitudinally sectioned fibres often seem to appear in a parallel set-up (Fig. 14a, 14c). Among the three areas the 1st one contains most matrix and therefore fibres are fewest present here. The antennules are predominantly surrounded by cement of the 2nd area and partly by cement of the 1st area. “Foot-like” elevations of the cuticle of the female are filled with cement (Fig. 10). Abrupt transitions between the three areas are seen (Fig. 10-12).

No regular layering can be seen both in the dwarf male of *Ibla cumingi* and the dwarf male and the cyprid of *Scalpellum scalpellum*.

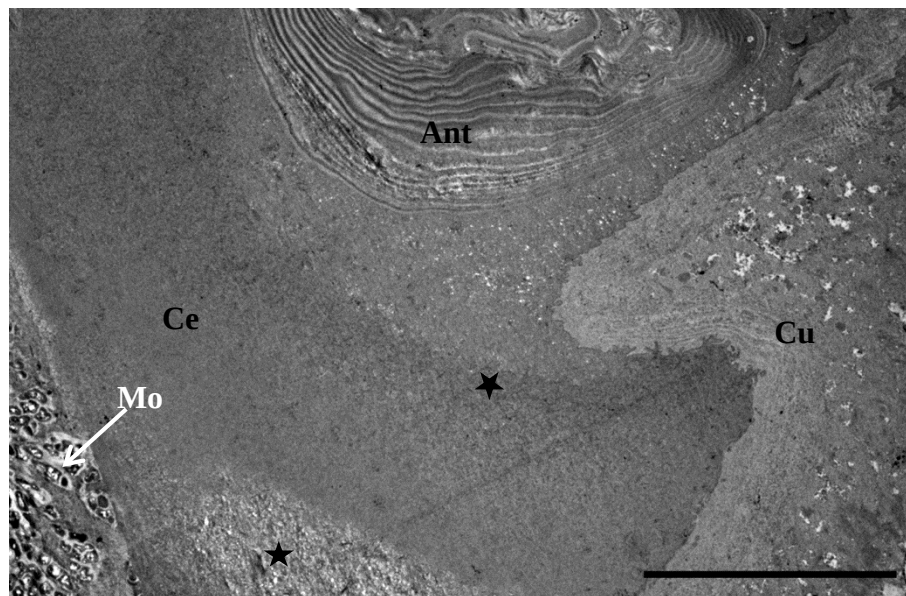


Fig. 9: *Ibla cumingi* dwarf male: overview of the cement (Ce). Asterisks indicate regions of the cement where electron-dense fibres are clearly visible. On the right side the cuticle of the female (Cu) is visible and on the top of the picture the cuticle of the antennule (Ant) of the male can be seen. At the bottom left are inclusions of microorganisms (Mo) in the cement. Scale bar: 10 μ m.

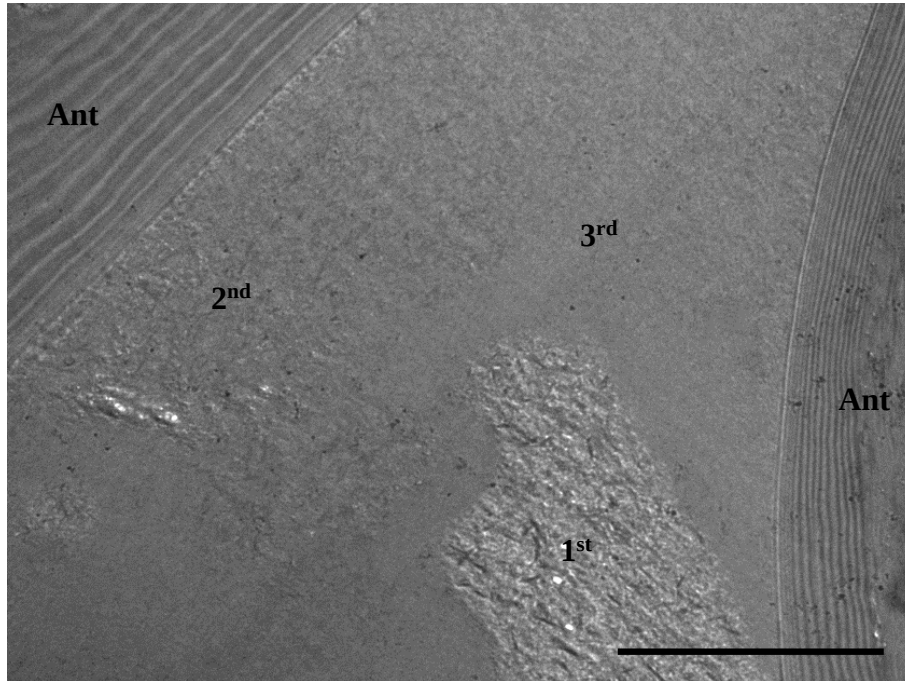


Fig. 10: 1st, 2nd and 3rd area of the cement of *Ibla* dwarf male. The transition between the 1st and 3rd area is abrupt. On the left and right side is the cuticle of the antennules (Ant) of the male. Scale bar: 5 μ m.

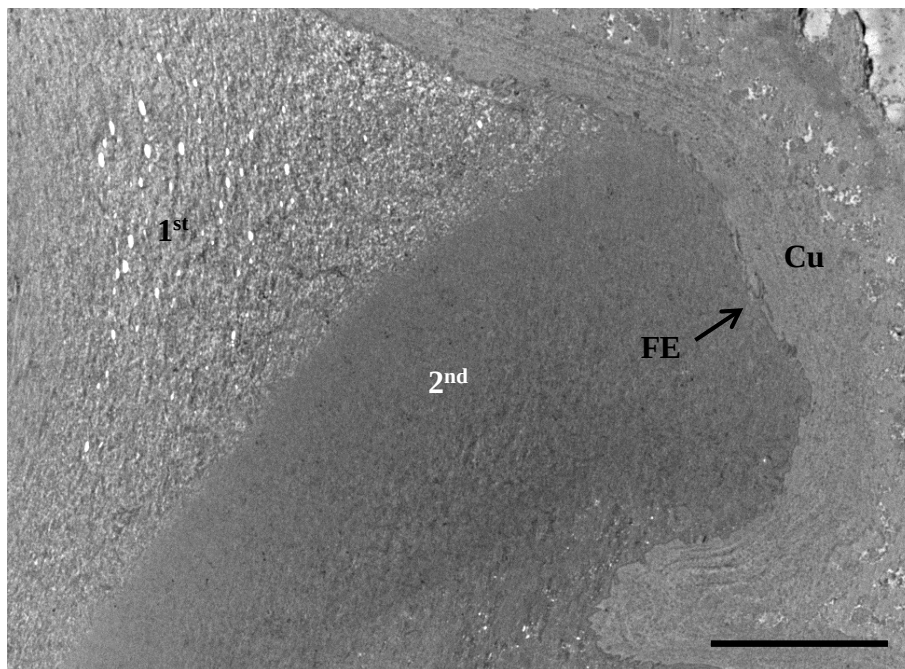


Fig. 11: *Ibla cumingi* dwarf male. Differently structured cement in 1st and 2nd area. The 1st area contains inhomogenously arranged fibres embedded in more electron-lucent matrix. The 2nd area contains more fibres that are homogenously distributed. The “foot-like” elevations (FE) of the cuticle of the female (Cu) are filled with cement. The transition between the two areas is abrupt. Scale bar: 5 μ m.

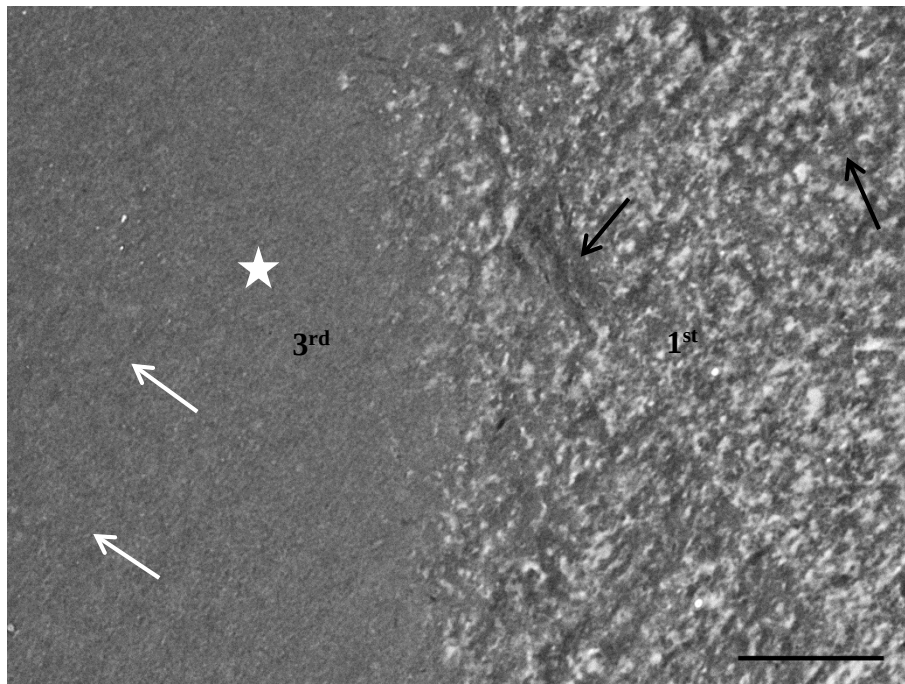


Fig. 12: Transition from area 3rd to 1st area in *Ibla* male. At the left side mainly cross sectioned fibres appear that are homogeneously distributed (asterisk). Also longitudinal sectioned fibres appear within this area (white arrows). At the right side electron-dense fibres are inhomogeneously arranged within a bright matrix. Some are forming thicker electron-dense conglomerates of fibres (black arrows). The transition between the two areas is abrupt. Scale bar: 1 μ m.

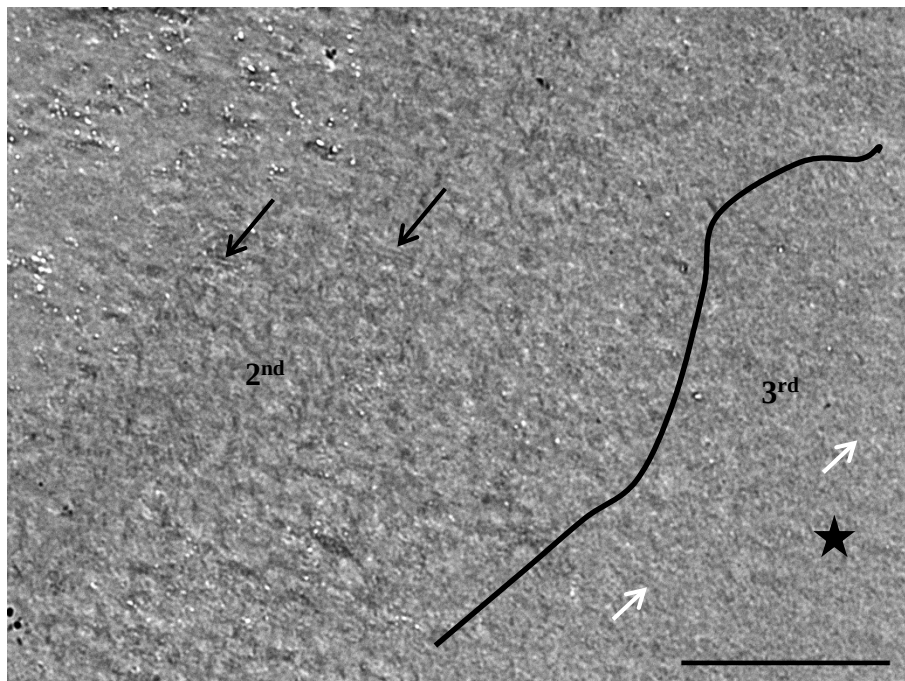


Fig. 13: Transition from 2nd to 3rd area in *Ibla* male. Arrows at the left side show longitudinally sectioned fibres of the 2nd area that seem to run parallel. The black line indicates the transition between the two areas. In the bottom right corner are mainly cross sectioned fibres (asterisk) of the 3rd area. But also longitudinally sectioned fibres are visible (white arrows). Scale bar: 2 μ m.

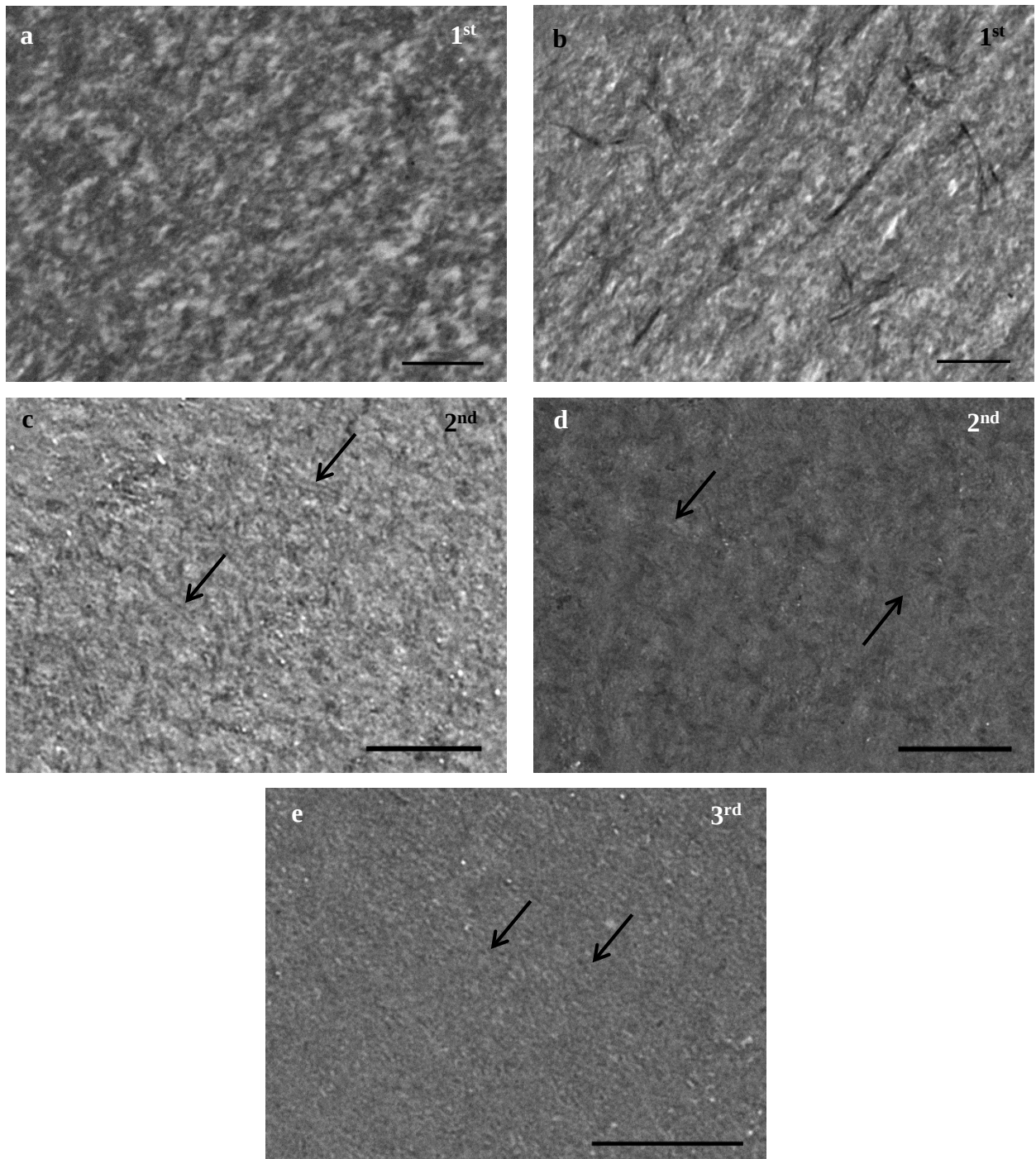


Fig. 14: Comparison of the different areas in the cement of the male of *Ibla cumingi*. **a, b)** 1st area: Inhomogenously arranged fibres within a bright electron-lucent matrix. **c)** 2nd area: Longitudinally sectioned fibres of the cement. Arrows point at fibres that seem to run parallel. **d)** 2nd area: Arrows point at very thin fibres appearing longitudinally sectioned. **e)** 3rd area. Arrows show cross sectioned electron-dense fibres homogenously distributed.. Scale bars a, b, e: 0.5 μm ; c, d: 1 μm .

4.1.2.4 The hermaphrodite of *Pollicipes pollicipes*

In contrast to the other species analysed the cement of *Pollicipes pollicipes* contains multiple layers mostly parallel arranged (Fig. 15, 16). These layers show different densities and orientation of the electron-dense fibres. Some layers appear fibrillar where fibres are mainly longitudinally sectioned. Others look granular that are cross-sectioned (Fig. 16). Within the cross sectioned fibres diverse electron-dense layers exist (black arrows and asterisks in Fig. 16a-d). In the different layers the fibres are orientated perpendicularly. Alternations of fibrillar and granular appearing sheaths exist (Fig. 16d). Like in the cement of the dwarf male of *Ibla cumingi* parallel arranged fibres also appear in *Pollicipes pollicipes* (Fig. 17a Insert). Yet also not parallel thick fibrillar layers were found (Fig. 17b). In addition a net-like structured fibrillar cement can be seen (Fig. 18). Electron-dense areas within the network can be seen in outlines. The boundary layer of the cement is rough and uneven.

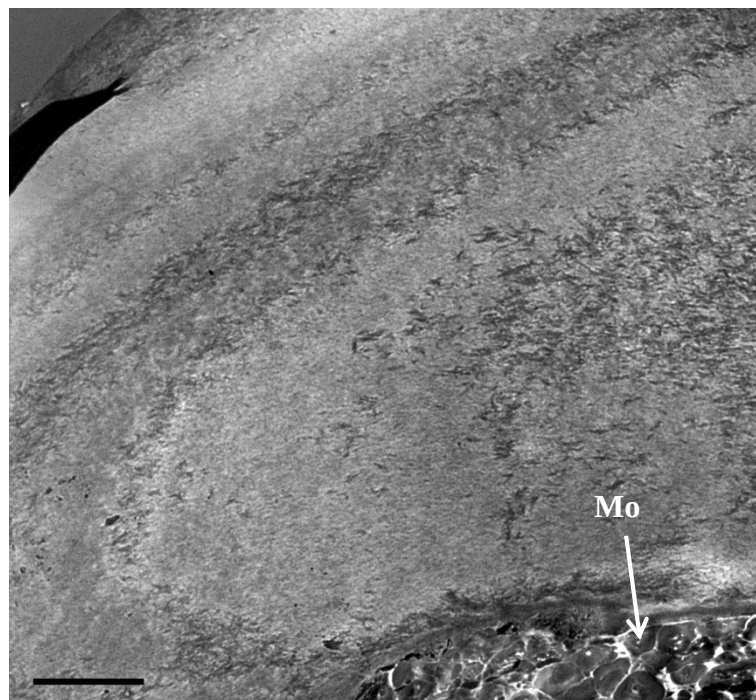


Fig. 15: Overview of layered cement with enclosed microorganisms (Mo) in the hermaphrodite of *Pollicipes pollicipes*. Scale bar: 2 μ m.

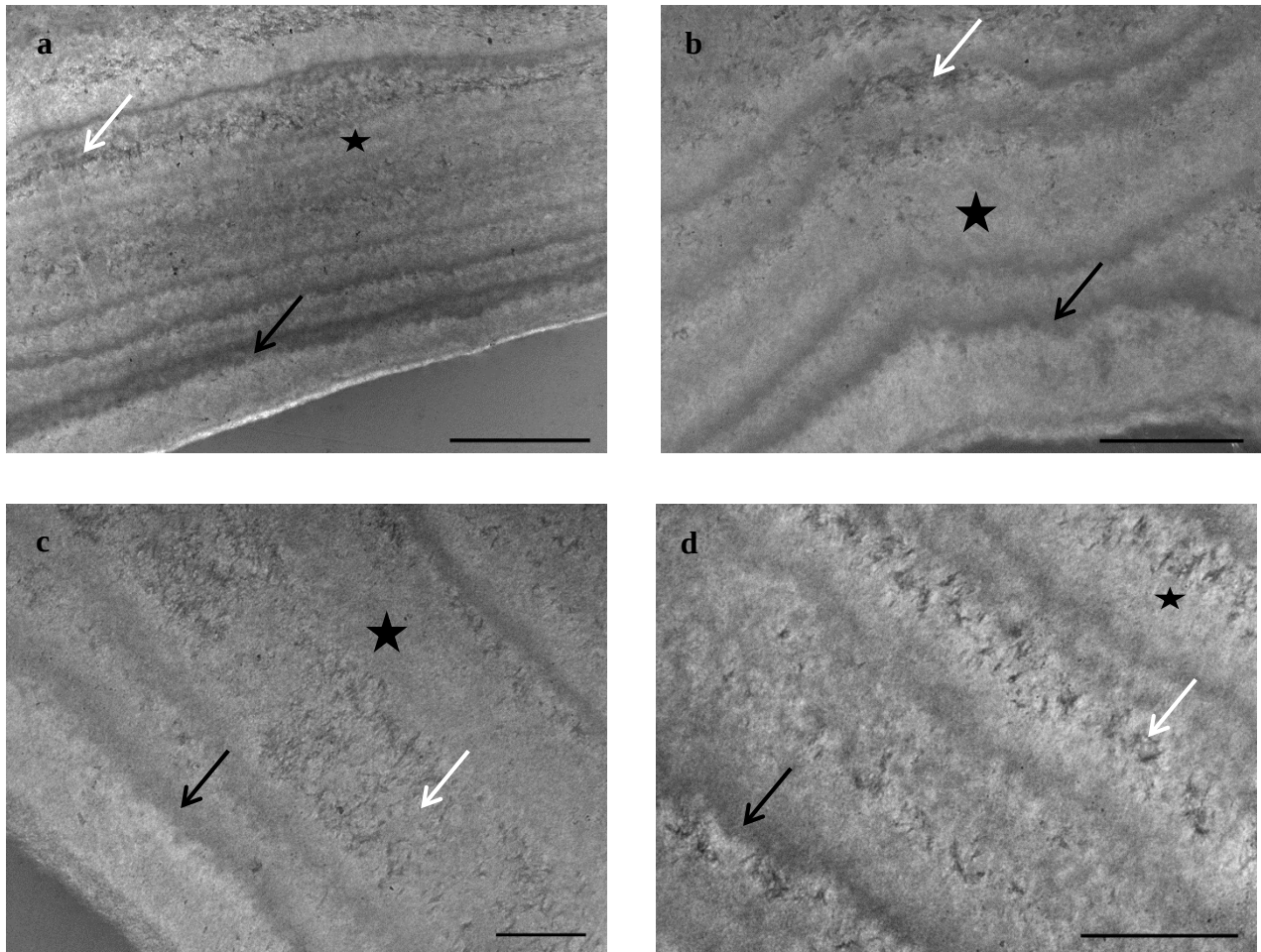


Fig. 16: a-d) Comparison of four areas of layered cement in *Pollicipes pollicipes* seen at different magnification. Multiple electron-dense layers of granular appearance (black arrows) alternating with electron-lucent layers (asterisks) and electron-dense fibrillar layers (white arrows) are visible. Scale bars a, b: 5 µm; c, d: 2 µm.

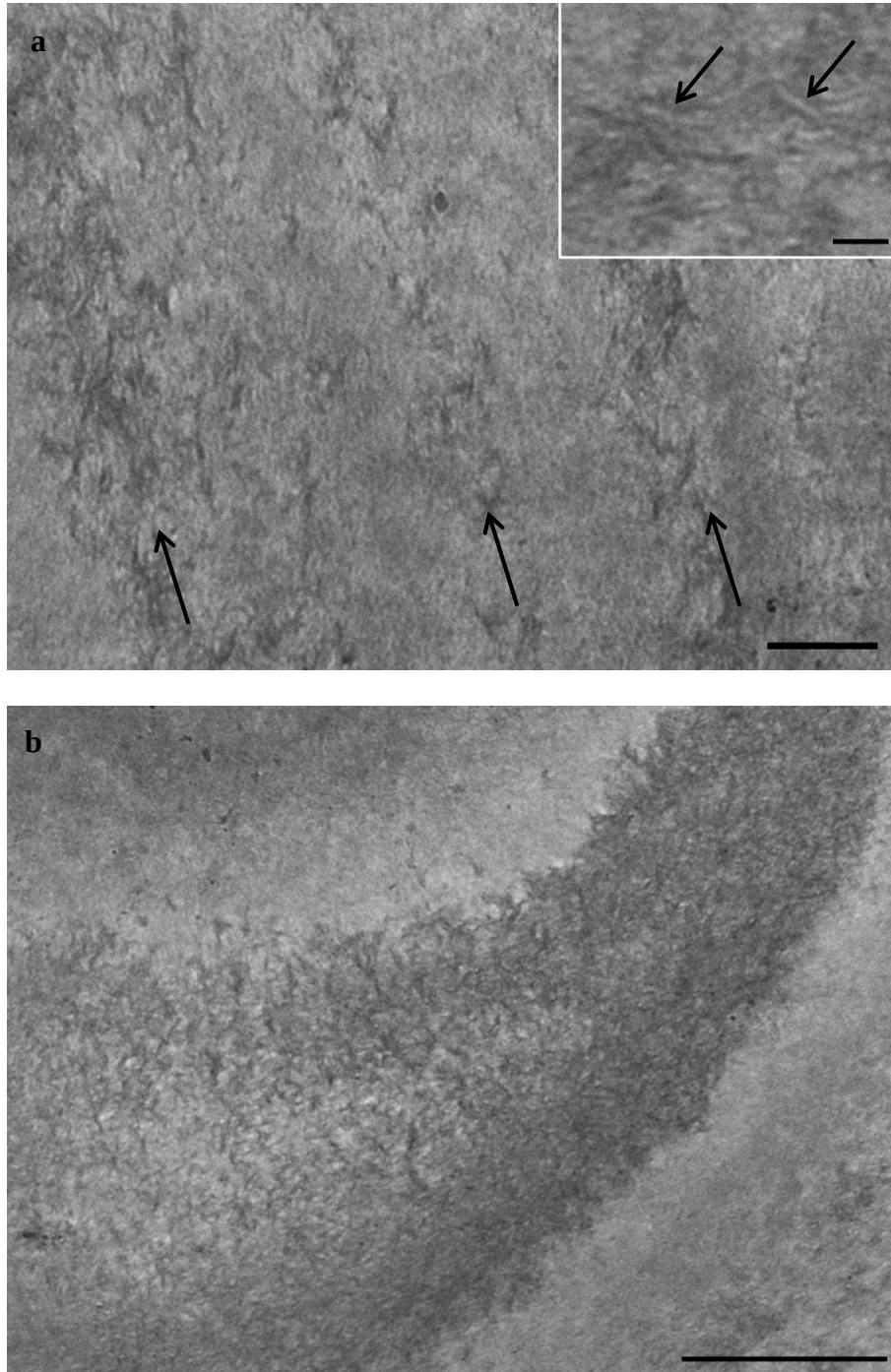


Fig. 17: *Pollicipes pollicipes*. **a)** Parallel electron-dense fibrillar layers (arrows) more detailed. Insert: Arrows show parallel set up of electron-dense fibres. **b)** This layer of electron-dense fibres is unlike the majority of the areas observed not a continuous uniform layer – the limiting borders do not run parallel. Scale bars a: 1 μm ; Insert a: 0.5 μm ; b: 2 μm .

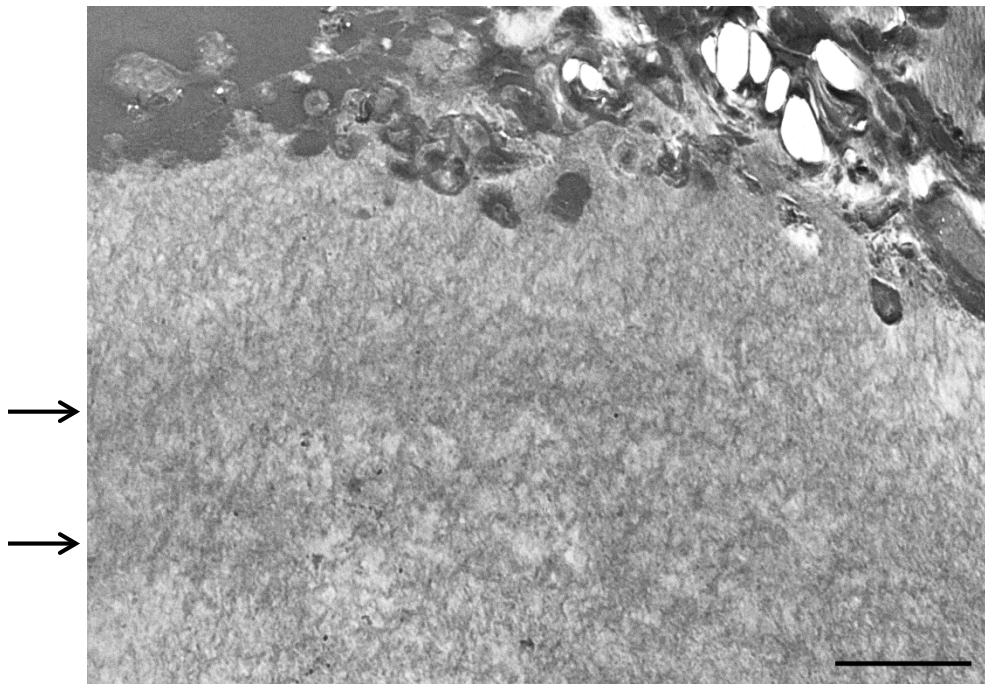


Fig. 18: *Pollicipes pollicipes*. Comparatively huge area of net-like fibrillar cement at the outer rim of the adhesive plaque. The arrows next to the picture indicate two layers of densely arranged fibres. The fibres at the outermost parts of the adhesive seem to be arranged less dense. Scale bar: 2 μm .

4.2 Thickness of fibres in the cement

By means of transmission electron microscopy the structure of the cement analysed here is shown as fibrillar. No measurements of the length of the fibres were made since no complete series of sections was available.

Concerning the thickness of the fibres in the cement measurements revealed the highest mean values in the dwarf male of *Scalpellum scalpellum* and its cypris larva (Tab. 2). The hermaphrodite of *Pollicipes pollicipes* and the dwarf male of *Ibla cumingi* have on average the same thickness of cement fibres. Even though the mean values of the fibre's thickness are very similar, the lowest and highest measured values diverge widely, most of all in the cyprid of *Scalpellum scalpellum* (14 nm – 57 nm). The range of the measured fibre-thickness in the dwarf male of *Ibla cumingi* was between 13 nm – 44 nm, in *Pollicipes pollicipes* between 9 nm – 50 nm and in the dwarf male of *Scalpellum scalpellum* between 20 nm – 50 nm.

Tab. 2: Comparison of the fibre thickness in the cement of *Scalpellum scalpellum* (cyprid: n=121; dwarf male: n=123), *Ibla cumingi* (n=272) and *Pollicipes pollicipes* (n=436). The stated results are the mean values with the standard deviation in nanometre.

	Species	Thickness of fibres [nm]
Cyprid	<i>Scalpellum scalpellum</i>	30 ± 8
	<i>Scalpellum scalpellum</i>	33 ± 6
Dwarf males	<i>Ibla cumingi</i>	25 ± 6
	<i>Pollicipes pollicipes</i>	25 ± 8
Hermaphrodite		

5. Discussion

5.1. Structure of the cement

There is big evidence that the structure of the cement depends on the substratum to which it is attached. This was shown by different studies that focused on acorn barnacles. Wiegemann & Watermann (2003) discovered that those growing on a low surface energy medium like polydimethylsiloxane (PDMS) produced a thick layer of cement. Berglin & Gatenholm (2003) described it as a softer adhesive than the adhesives from barnacles growing on a medium surface energy substratum like polymethylmethacrylate (PMMA).

Also the optical image with light-microscopy demonstrated a different appearance of the adhesive depending on the subsurface. So it appeared clear on PDMS whereas structured on PMMA. And more adhesive was produced on PDMS (Berglin & Gatenholm, 2003).

According to these results it is obvious that the three species analysed in this study show different structured cement because they settle on different substrata. *Pollicipes pollicipes* grows on rocks (Barnes, 1996) the dwarf male of *Ibla cumingi* in the mantle cavity of the female (Klepal, 1985), and the cyprid and the dwarf male of *Scalpellum scalpellum* in the receptacles at the rim of the mantle aperture of the hermaphrodite (Spremborg et al., 2012).

A clear zonation of alternating layers could only be seen in the cement of *Pollicipes pollicipes*. But still the fibres are also arranged in a net-like manner as shown in Fig. 18 of *Pollicipes pollicipes*. Though, it cannot be alleged that the adhesives of the dwarf males of

Ibla and *Scalpellum* and its cyprid are homogenous masses. They either show fibres of different density as in *Scalpellum scalpellum* or several patches of differently orientated fibres within the adhesive plaque as seen in the dwarf male of *Ibla cumingi*. Several studies revealed zonation of the cement in different species of Thoracica.

Zheden et al. (2012) found out that the cement of the stalked barnacle *Dosima fascicularis* has concentric layers of cement around the stalk. Further the adhesive is described as fibrillar and condensed zones forming borders to the bubbles within the cement are seen. Wiegemann & Watermann (2003) show in the cement of *Balanus improvisus* growing on PDMS about 15 layers of reticulated adhesive parallel to the substratum. The cement of *Balanus improvisus* growing on conspecifics however shows no layers and it is a dense thin sheath (Wiegemann & Watermann, 2003). Walker (1971) presents the zoned cypris cement of *Balanus balanoides*. The adhesive, attached to plant-tissue, is reticulated and forms three layers of different electron-density. Walker's explanations for the zonation are that sea water might influence the outer layers of the adhesive, or tanning processes in the cement might cause layers by linking with e.g. oxygen. Gruber (2011) did not only find fibrillar and homogenous electron-dense cement in *Semibalanus balanoides* but also zonation within the adhesive. These zones contain the same structural material and are separated by an electron-dense line. This author suggests that the differently structured zones can be the result of different periods of cement secretion. This theory is supported by the investigations of Fyhn & Costlow (1976) who found out that the cementing process takes place during the intermolt-cycle of the barnacles. Each cement layer can therefore indicate one molting-cycle. But the absence of concentric layers in the cement of the dwarf male of *Ibla cumingi* and the dwarf male and cyprid of *Scalpellum scalpellum* are most likely associated with their sheltered way of life in contrast to the exposed life of *Pollicipes pollicipes*.

The fibrillar cement of *Scalpellum scalpellum* looks like a 3D-network. It is obvious that the images shown are in a negative contrast. It is very likely that this effect happened due to the long storage of the samples in formalin.

Due to the nano-sized fibres that are embedded in the matrix it can be considered that the cement is a fibre-reinforced composite material – or rather a “nanocomposite” (Gibson, 2012). The idea of the cement as a composite material also emerges from the study of Sullan et al. (2009), where the cement was split into a structural or mesh-like component (built up of fibres, globules and rod-shaped structures) and a non-fibrillar component – the matrix.

Different types of composites according to the orientation of fibres and the arrangement of layers exist, each with its specific properties (Gibson, 2012). So it can be assumed that the differences found in the cement analysed imply different physical properties that are a consequence of the various environmental conditions the animals’ adhesives have to cope with such as substratum, depth, water-movements and even perhaps sunlight. It is obvious that only the cement of *Pollicipes pollicipes*, living in the surf, shows a clear zonation, compared to the cement of the other species analysed that live protected. Further on *Pollicipes pollicipes* is the only one of the species analysed where the ability of active relocation is known (Kugele & Yule, 2000).

It may be that following the systematic positions of the species analysed (Pérez-Losada et al., 2008) also the structure of the cement of the *Ibla cumingi* dwarf male presents a simpler pattern compared to the dwarf male and cyprid of *Scalpellum scalpellum* and the hermaphrodite of *Pollicipes pollicipes*. This would be obvious according to Lacombe (1970) where she supposes that the arrangement of the cement glands resembles the systematic position. Otherwise, this cannot be supported by the results of the measured fibre thickness where of all things the mean values of *Ibla* as a basal and *Pollicipes* as a more evolved representative are the same and *Scalpellum* as in between, has higher values.

Consequently, the fibres embedded in the matrix of the cement are a basic structure of the barnacle adhesive. Differences in this structure are expected in the proximate level, e.g. in the formation of layers.

“Foot-like” elevations of the cuticle as mentioned by Gruber (2011) are also present in the studied samples of the dwarf male and cyprid of *Scalpellum scalpellum* and the *Ibla cumingi* dwarf male. One theory is that the filling of small indentations with cement helps the barnacles additionally adhere to the substratum (Wiegemann, 2005) or enlarges the adhesion strength (Berglin & Gatenholm, 2003). Though this argument is stated for acorn barnacles it is most likely that the dwarf males of stalked barnacles – as used in the present study - also use this kind of nano-anchoring.

The occurrence of micro-organisms like algae or bacteria within the cement has also been described previously (Gruber, 2011; Wiegemann & Watermann, 2003; Zheden et al., 2012).

5.2. Thickness of fibres in the cement

The difference in thickness of the fibres of the dwarf male and the cypris cement of *Scalpellum scalpellum* can be explained by the fact that cypris cement differs from adult cement in the way of production (Kamino & Shizuri, 1998; Walker, 1971). Despite the evidence that also the morphology of the adhesive differs in cyprids (Aldred et al., 2008) and adults (Berglin & Gatenholm, 2003; Wiegemann & Watermann, 2003) the results from *Scalpellum scalpellum* in the present study cannot support this theory. Because of similar structures of the adhesives (of the cyprid and the dwarf male), the theories about influence of the substratum, on which the animal settles on, to the morphology of the cement are more likely (Berglin & Gatenholm, 2003).

The big differences in the lowest and highest values of fibre thickness can be explained because of overlapping or merged fibres (Wiegemann & Watermann, 2003). But in contrast to the measured fibres of the acorn barnacle *Balanus improvisus* that are from 1 µm up to 50 µm in AFM (Wiegemann & Watermann, 2003) the measured fibres in the present study of stalked barnacles are between 9 nm and 57 nm in TEM. Wiegemann & Watermann (2003) have other results with SEM where they found much smaller fibre-diameters down to about 20 nm,

comparable to the values in the present study. But the reliability of these low values found with SEM is questioned by the authors themselves. Dehydration during the preparation process for SEM could have had effects on the fibre diameter. Compared to other investigations the measured diameters of the fibres in this study are at the lower limit.

The study of Wiegemann & Watermann (2003) revealed that the adhesive fibres are composed of merged granules with a diameter of about 50 nm. The presence of granular components in the cement is also shown by Berglin & Gatenholm (2003) with AFM in *Balanus improvisus*. But they measured much bigger granules (between 84.2 ± 2.5 nm and 74.8 ± 2.2 nm). On the basis of AFM analysis Sullan et al. (2009) report different sized structures in the cement of *Balanus amphitrite* such as “clustered globules” with 60-100 nm, “small globules” of 10-30 nm and “rod-like” structures with 11 nm in diameter and 300 nm length. Due to incomplete series of sections measurements of the length of the fibres in the species analysed in this study were not possible.

All those fibrillar and globular units can merge and form several bigger structures such as “net-like” and “sponge-like” structures, globules can form dense or loose layers (Wiegemann & Watermann, 2003). The electron-dense dots that are described on sections of the cement of *Pollicipes* and the dwarf male of *Ibla* can also be globular structures. But the measured diameters of those dots are within the range of the diameters of the fibres so it is more likely that they are cross sections of the fibres. The fibres described as “parallel” might be straight portions of spirals.

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7. Zusammenfassung

Der proteinreiche Zement der Cirripedia besteht aus einem fasrigen Anteil der in eine Matrix eingebettet ist. Diese Tatsache lässt die Vermutung über den Zement als Faserverstärkten Verbundwerkstoff (Gibson, 2012; Sullan et al., 2009) zu. Vorangegangene Studien haben die Durchmesser der Fasern und globulären Strukturen des Zements zwischen 11 nm und 50 µm gemessen (Berglin & Gatenholm, 2003; Sullan et al., 2009; Wiegemann & Watermann, 2003). Es wurde berichtet, dass sich diese Strukturen zusammenschließen und aneinander legen können und z.B. „netzartige“ oder „schwammartige“ Strukturen bilden. Darüber hinaus zeigten Studien die Abhängigkeit der Struktur des Zements vom Substrat (Berglin & Gatenholm, 2003; Wiegemann & Watermann, 2003).

Bisher wurde die Struktur des Zements von Cirripeden hauptsächlich an Sessila untersucht. Die dabei verwendete Methode beschränkte sich weitgehend auf Atomic Force Mikroskopie (AFM) und Raster Elektronen Mikroskopie (REM). Nur wenige Autoren haben Untersuchungen des Adhesivs an pedunculaten Cirripeden mit dem Transmissions Elektronen Mikroskop (TEM) durchgeführt (Zheden et al., 2012).

Das Ziel dieser Diplomarbeit war die Struktur des Zements von drei gestielten Cirripeden, der Cypris-Larve von *Scalpellum scalpellum* (LINNAEUS, 1767), den Zwergmännchen von *Scalpellum scalpellum* und *Ibla cumingi* (DARWIN, 1851), sowie dem Hermaphroditen *Pollicipes pollicipes* (GMELIN, 1790), vergleichend zu untersuchen. Die dabei verwendeten Methoden waren Transmissions Elektronenmikroskopie (TEM) und Lichtmikroskopie (LM).

Die Dicke der in die Matrix des Zements eingebetteten Fasern wurde gemessen und liegt zwischen 9 nm und 57 nm. Die große Spannweite zwischen den Minima und Maxima kann damit erklärt werden, dass sich mehrere dünne Fasern aneinander legen und stärkere „Faserbündel“ bilden. Es wurde festgestellt, dass die elektronen-dichteren Fasern des Zementes unterschiedlich orientiert in der Matrix eingebettet sind. Bei der Cypris und dem Zwergmännchen von *Scalpellum scalpellum* kann zwischen dicht- und locker angeordneten Fasern unterschieden werden. Im Zement des Zwergmännchens von *Ibla cumingi* werden drei Zonen unterschieden. Die 1. Zone besteht aus ungleichmäßig verteilten elektronen-dichten

Fasern. In der 2. Zone finden sich gleichmäßig verteilte elektronen-dichte Fasern die längs angeschnitten sind. Und die 3. Zone zeichnet sich durch elektronen-dichte Punkte aus. Da die Querschnitte der punktförmigen Strukturen mit den Fasern der anderen Zonen übereinstimmen, liegt die Vermutung nahe, dass es sich um quergeschnittene Fasern handelt. Es wäre aber auch möglich, dass diese punktförmigen Erscheinungen kugelförmige Strukturen sind, da in Wiegemann & Watermann (2003) die Zusammensetzung der Fasern aus globulären Einheiten beschrieben wird. Der Zement von *Pollicipe pollicipes* zeigt eine deutliche Schichtung die als Abfolge von orthogonal zueinander orientierten Fasern zu verstehen ist.

Die Unterschiede im Zement der untersuchten Arten können durch die verschiedenen Substrate auf denen sie wachsen, erklärt werden. Denn das Zwergmännchen von *Ibla cumingi* wächst auf Cuticula des Weibchens (Klepal, 1985), die Cypris und das Zwergmännchen von *Scalpellum scalpellum* leben auf Gewebe des Hermaphroditen von *Scalpellum scalpellum* (Spremborg et al., 2012) und der Hermaphrodit *Pollicipes pollicipes* wächst auf Felsen (Barnes, 1996). Zusätzlich müssen auch die unterschiedlichen physikalischen Bedingungen, denen die Tiere ausgesetzt sind, berücksichtigt werden. So leben die Cypris und das Zwergmännchen von *Scalpellum scalpellum* und das Zwergmännchen von *Ibla cumingi* geschützt in den „Receptacles“ des Hermaphroditen beziehungsweise in der Mantelhöhle des Weibchens, während der Hermaphrodit *Pollicipes pollicipes* den mechanischen Beanspruchungen in der Brandung ausgesetzt ist. Die unterschiedliche Orientierung der Fasern im Zement kann auf diese unterschiedliche mechanische Beanspruchung hindeuten.

Die beiden Arten, *Ibla cumingi* und *Pollicipes pollicipes*, die phylogenetisch am weitesten auseinander liegen (Pérez-Losada et al., 2008), haben dieselben Faserdurchmesser. Die Cypris und das Zwergmännchen von *Scalpellum scalpellum*, phylogenetisch zwischen *Ibla cumingi* und *Pollicipes pollicipes*, haben um 5 - 8 nm dickere Fasern. Auffällig ist, dass nur der Zement von *Pollicipes pollicipes*, als Bewohner der Brandungszone klar geschichtet ist.

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DANKE!

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